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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C12N 9/18, 1/20, C12Q 1/44 A61K 37/54, C12P 17/18 C12M 1/40 // (C12N 1/20 C12R 1:38)

A1

(11) International Publication Number: WO 93/02186

(43) International Publication Date: 4 February 1993 (04.02.93)

(21) International Application Number: PCT/GB92/01324

(22) International Filing Date: 20 July 1992 (20.07.92)

(30) Priority data:
9115765.1 22 July 1991 (22.07.91) GB

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(81) Designated States: JP, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE).

**Published**
*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: BACTERIAL ESTERASE FOR COCAINE DETECTION

(57) Abstract

A cocaine esterase has been isolated from a strain of the bacteria *Pseudomonas maltophilia*. The cocaine esterase catalyses the debenzoylation of cocaine. This reaction may be used in the detection of cocaine. The enzyme may be incorporated into sensors for this purpose.

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# BACTERIAL ESTERASE FOR COCAINE DETECTION.

## Background of the invention

This invention relates to a new enzyme isolated from a microorganism, the microorganism that produces this enzyme, the use of this enzyme in catalysing the degradation of cocaine and a method and apparatus for the detection of cocaine using this enzyme.

## Description of the prior art

There is an urgent need for a better method of detection of cocaine in particulate form and in body fluids. In relation to particulate cocaine, although many different analytical systems have been proposed, most are based on large pieces of equipment such as mass spectrometry, and require specially trained laboratory technicians. Such systems include thin layer chromatography, gas chromatography and high pressure liquid chromatography (HPLC). In relation to body fluids they require extraction of the sample to remove interfering compounds. Labelled assays have been used but these also require specialist skills to carry out. Some of the above or other prior methods, e.g. mass spectrometry, also require bulky and expensive equipment.

Portable detecting devices for cocaine have recently been developed. Thus, Einceman et al. (Anal. Chem. (1990) 62 1374-1379) have developed a portable ion mobility spectrometer, but it relies heavily on the volatility of the drug. A similar drawback exists with the piezoelectric crystal coated detecting device of Ngeh-Ngwainbi et al. (Biosensors & Biomechanics (1990) 5 13-26). A potentiometric sensor has been developed by K. Vytras et al., (Mikrochimica Acta (1984) [Wein] III 139-148) but this is very unspecific as it forms a complex with the tertiary amine group present in many illicit drugs.

## Summary of the invention

We have now found an enzyme which can be used in the detection of cocaine. The enzyme is a cocaine esterase (hereinafter CE) which catalyses the debenzoylation of cocaine

that is to say its hydrolysis to produce ecgonine methyl ester and benzoic acid. This enzyme could thus be used to detect cocaine, by reacting the enzyme with cocaine and detecting the occurrence of the enzyme-catalysed reaction. One such method of detection would be conductimetric, as the CE reaction will bring about a change in conductance of the solution. Alternatively, the method of detection could be potentiometric, the CE reaction bringing about a change in the pH of the solution. Other methods could include optical, colorimetric, thermometric or amperometric detection of the reaction products, such methods being well known in the art. Accordingly the invention includes sensors, especially of the conductrimetric or potentiometric type for cocaine esterase. These and similar sensors can be used as the basis for convenient portable sensors for detecting cocaine in body fluids, luggage, clothing etc. of smugglers, traffickers and cocaine users. Accordingly the invention provides an important advance in the fight against and control of use of drugs.

The term "cocaine" used throughout the specification comprises the free base and salts thereof, unless the context requires a more specific meaning.

In a first aspect the invention provides the cocaine esterase enzyme. The cocaine esterase enzyme catalyses the debenzoylation of cocaine into ecgonine methyl ester and benzoic acid; thus it attacks the benzoate ester linkage of cocaine. There is little or no activity at the methyl ester linkage. Thus, ability to specifically attack the benzoate ester linkage of cocaine is a distinctive feature of the cocaine esterase of the invention, that is not possessed by commercially available esterases, e.g. porcine liver esterase, horse serum butyryl-cholinesterase or other esterases such as microbial atropinesterase.

Another distinctive feature of the CE is that it has a native molecular weight in its unaggregated form of about 120,000 Daltons and when aggregated of about 420,000 Daltons (both as determined by elution from a gel filtration column calibrated with protein markers). By the term "about" we mean to encompass

variations which are usual in the determination of high molecular weights by this method and certainly to include a variation of up to 10%. This molecular weight is considerably different from that of microbial atropinesterase which has a molecular weight of 30,000 Daltons when denatured and 60,000 Daltons in its native form (Rorsch et al., Proc. K. Ned. Akad. Wet. (1971) Ser. C 74 132-152).

Example 3 hereinafter describes other features of the CE, but it is expected that it will be possible to vary some of these by changing the conditions of growth of the microorganism which produces it, or by a higher degree of purification of the enzyme. Accordingly, it is not preferred to rely on such characteristics as the catalytic activity or the thermal stability of CE in the most general definition of the enzyme. Any one, or more of them can be permitted (as the context permits) as alternative ways of defining the enzyme, but they are best seen as one or more preferred, additional characteristics to one or more of those defined above.

The CE is obtained from a bacterial strain isolated from nature. The bacterial strain is a strain of Pseudomonas maltophilia herein designated "MB11L". A Budapest Treaty patent deposit of this bacterium has been made at the National Collections of Industrial and Marine Bacteria (NCIMB), 23 St. Machar Drive, Aberdeen, AB2 1RY, Scotland on the 14th June 1991 under the deposit number NCIMB 40427. Considerable difficulty lies in the exact taxonomical classification of some members of Pseudomonas maltophilia as they could equally be classified as Xanthomonas maltophilia. Pseudomonas has been preferred in this instance but this organism may also be classified within the genus Xanthomonas. This bacterium, together with mutants and variants producing the CE of the invention, are included in the present invention. The CE can be produced by culturing such a bacterium on a source of carbon and nitrogen.

Brief description of the drawings

Figures 1 to 4 plot the course of important stages in the

chromatographic purification of the CE (see Example 2).

Description of the preferred embodiments

Cocaine esterase can be produced by culturing P. Maltophilia on a source of carbon and nitrogen. Preferably the source is cocaine itself. When grown on glucose or benzoate the activity of the CE is lower than when grown on cocaine. Additionally D,L-methionine is preferably added to the growth medium as this increases the specific activity of the cocaine esterase produced. In an alternative, an equimolar mixture of cocaine and glucose yields an enzyme of high activity. Cultivation is preferably aerobic at any usual temperature, e.g. within 20 to 40°C range, preferably 25 to 35°C. To obtain the enzyme the cells can be disrupted in any conventional way. Preferably a cell-free extract is made. The enzyme is then recovered from the cells or the extract.

Instead of the precise starting organism deposited, a mutant thereof, e.g. derived by gamma-ray irradiation or use of a chemical mutant, induction by culture on another medium etc. or a transconjugant thereof with another bacterium or an artificially produced variant can be used.

The enzyme or some modification thereof can also be made by recombinant DNA technology using methods well recognised in that art. These may entail producing the enzyme in another host organism.

The invention is particularly applicable to the detection of grains of powdered cocaine (free base or any of its salts) in luggage, cargo, or about the person, or for the detection of cocaine or its metabolites in biological fluids especially in urine and blood.

The enzyme of the present invention is of use primarily in the detection of cocaine. This may be achieved by the use of cocaine esterase enzyme alone or in conjunction with one or more other enzymes that will further improve the detection of cocaine.

The methods of making biosensors that rely on conductimetric, amperometric or potentiometric changes in the test reaction are well known in the art. UK Patent Application Publication No.

2,231,332A (NRDC) describes such methods and their use in biosensors, the contents of which in relation thereto is hereby incorporated by reference.

Any of such methods may be of use in detecting the occurrence of the cocaine esterase reaction and hence in the detection of cocaine.

The cocaine esterase of the invention has several other uses. It can be used to "clear" the body of excess cocaine after a cocaine overdose. Similarly the organism MB1lL can be used to "clear" industrial waste of cocaine. The term "clear" is used to imply the removal of cocaine to its less addictive metabolites. Thus the cocaine esterase enzyme of the present invention has a use in therapy. Also the enzyme can be used to separate cocaine isomers and under the correct conditions, to form cocaine analogues by the reversal of the debenzoylation reaction (biotransformation).

The following Examples illustrate the invention. "TRITON", "SEPHACEL" and "SEPHACRYL" are Registered Trade Marks.

EXAMPLE 1

Preparation of a cocaine esterase from the bacterial strain Pseudomonas maltophilia MB1lL.

Materials and Methods

1. Pseudomonas maltophilia (MB1lL) was isolated from samples collected from a natural source by enrichment with cocaine as the sole carbon source.

MB1lL was grown in 750ml of defined medium consisting of $Na_2HPO_4$ (4.33g), $KH_2PO_4$ (2.65g), $NH_4Cl$ (2.0g), $N(CH_2CO_2H)_3$ (0.1g) per litre, containing 4ml/l of a mineral salt solution as described by Rosenberger and Elsden (J. Gen. Microbiol. (1960) 22 726-739). Cocaine (90% pure w/w) was added aseptically as the sole carbon source (10mM) to 750ml of the above sterile medium in a 2 litre Ehrlenmeyer flask and shaken at 250 rev/min in a shaking incubator. For bulk preparation of bacteria, 750ml of seed culture was asceptically added to a 10 litre culture vessel, containing 9.5 litre of sterile medium. The bulk cultures were

incubated at 30°C, stirred at 500 rev/min with sterile aeration at 18 l/min. The growth medium for the bulk culture was 10mM of said cocaine with the addition of 40 mg/l D,L-methionine and 700µl Antifoam A emulsion (Sigma), which is a 30% aqueous emulsion of silicone polymers.

Cell-free extracts were prepared from cells grown in the above manner. If cells were to be harvested from a 10 litre bulk culture, prior concentration of the cell broth to 2-3 litres was performed in a rotary concentrator fitted with a 200 sq cm membrane of 0.45µm pore size. The cells were then washed with 1 litre of growth medium. These cells or those obtained directly from a smaller volume culture were then pelleted by spinning at 10,000g for 15 min at 4°C in a Sorvall RC-5C centrifuge fitted with a GS-3 rotor. These pelleted cells were resuspended in 2ml of 50mM MOPS buffer (pH7), per gram wet cell weight. Cells were disrupted by sonication in an MSE Soniprep (Fisons Instruments, FSA Ltd.) using 18 x 12µm burst of 15 seconds, alternated with 30 seconds of cooling in melting ice. Cell debris and unbroken cells were removed by centrifugation at 48,000g for 20min at 4°C in a Sorvall RC-5C centrifuge using a SS-34 rotor, to give clarified cell-free extract.

2. Chemicals

Cocaine free base was obtained by dissolving the hydrochloride (5g, 15mmoles) in a minimum volume of distilled water, followed by the dropwise addition of 1.5ml of 10M sodium hydroxide solution. The resulting free base was extracted into 10ml diethylether and recovered by evaporation. A typical yield was 80%, melting point 96.4-98°C.

The resolution and identification of cocaine and its breakdown products, and benzoic acid was determined by HPLC analysis at 218nm or 275nm on Waters 600 system. The 0.46x 25cm column contained 5µm Spherisorb (C-18). The mobile solvent phase was as described by Noggle & Clark (J. Assoc. Off. Anal. Chem. (1982) 65 756-761) and Masoud and Krupski (J. Anal. Toxicol. (1980) 4 305-310). The mobile phase was sparged with helium at

30ml/min throughout operation.

3. Buffers

The following buffers were used in the purification and characterisation of cocaine esterase.

Buffer A : 50mM MOPS, 2% glycerol (v/v), 1mM β-mercaptoethanol, pH 7.0.

Buffer B : 50mM MOPS, 0.5% (w/v) cholate, 0.1M NaCl, 2% (v/v) glycerol, 1mM β-mercaptoethanol, pH 7.0.

Buffer C : 50mM sodium borate, 0.5% (w/v) cholate, 0.6M NaCl, 2% (w/v) glycerol, 1mM β-mercaptoethanol, pH 9.0.

Buffer D : 10mM $K_2HPO_4$ 0.5% (w/v) cholate, 0.1M NaCl, 2% (v/v) glycerol, 1mM β-mercaptoethanol, pH 6.8.

4. Assays

Cocaine esterase

Cocaine esterase in extracts, column fractions and characterisation experiments (e.g. pH optimum, Michaelis constant determinations) was routinely assayed by shaking incubation (250 rpm, 30°C) of 2mM cocaine HCl (made up in 1ml 50mM MOPS buffer, pH 7.0) with a known quantity of sample (10-200µl, 0-0.01U) for a fixed length of time (10-30 min). The reaction was stopped by the addition of 10µl of concentrated phosphoric acid and protein pelleted at 13,000 rpm in a minifuge. The amount of benzoic acid produced was determined by HPLC analysis of 50µl of the supernatant and comparison to standard samples (0-1mM) treated identically. Standard curves of peak height plotted against benzoic acid concentration were linear over the range 0-1mM. Controls containing no enzyme solution were run to allow for background hydrolysis. An assay was considered invalid if no cocaine remained at the end of the assay (seen by HPLC). One unit of esterase activity is defined as the amount of enzyme required to produce 1µmol of benzoic acid in 1 min at 30°C.

The presence of esterase in a sample could be detected in a similar fashion using gas chromatography to observe benzoic acid production, but no attempt to quantify the assay was made.

Gas chromatography was used in these Examples, but as

previously discussed, is an impractical tool for routine portable measurement of cocaine or cocaine esterase.

All spectrophotometric assays were performed on Perkin Elmer Lambda 3, 5 or 7 Dual Beam Spectrophotometers fitted with chart recorders and constant temperature cell jackets. Readings were against appropriate blanks.

Protein

Protein was routinely assayed by the Coomassie dye-binding method of Bradford (Anal. Biochem. (1976) 72 248-254) using commercially available reagent and Bovine Serum Albumin standard (Pierce Ltd. - obtained through Life Science Labs Ltd., Luton). An aliquot (20µl) of sample containing 0.2-1mg protein/ml was added to 1ml of reagent and the reaction allowed to develop for 5 min at room temperature prior to reading the absorbance at 595nm against a blank of buffer (20µl) plus reagent (1ml). Comparison to a standard curve of standard values (0-1mg/ml) allowed calculation of the protein concentration in the sample.

Gel filtration standards

The following enzymes were used as molecular weight markers in gel filtration experiments: Bovine liver catalase, yeast alcohol dehydrogenase and yeast C300 hexokinase (molecular weights 240,000, 150,000, 100,000 Daltons respectively). Assays for their activity were as described in Bergmeyer ((1986) Methods of Enzymatic Analysis. 3rd Edition, published by V.C.H. Publishers, Weinheim, Germany). Cytochrome c (molecular weight 13,000 Daltons) was detected by virtue of its absorbance at 505nm.

EXAMPLE 2

Purification of cocaine esterase

All steps were carried out at 4°C unless otherwise stated.

A cell free extract of P. maltophilia MB11L was prepared by sonicating and clarifying in cholate-free buffer A. Cholate was subsequently added to a concentration of 0.5% (w/v). Prior to DEAE-Anion Exchange Chromatography, the pH was checked and NaCl added to give a final conductivity of 13mS/cm. The final salt concentration typically approached 0.1M, but was carefully

controlled to ensure binding to DEAE Sephacel .

1. DEAE-Sephacel Chromatography

Cell free extract, typically containing 900mg of solubilised protein was loaded on to a 27 x 2.6cm DEAE-Sephacel (Pharmacia/ LKB Biotechnology Ltd.) column pre-equilibrated in buffer B. Loading, washing and elution of the column was at 15 ml/sq.cm/h. After washing with buffer B until no further elution of protein was detected at 280 nm, the cocaine esterase was eluted with a combined salt and pH gradient 125ml each of buffer B to buffer C followed by a wash of 100ml buffer C. Eluting protein was collected as 10ml fractions which were assayed for cocaine esterase activity and protein. (As shown in Fig. 1, the enzyme eluted at approximately 0.4M NaCl, pH 8.3. In this and each of the following figures esterase activity is denoted by filled circles and protein content by the dotted line. In Figs. 1 and 2 the dashed line represents the elution gradient). Active fractions were pooled and dialysed against 2 litre quantities of buffer D. (The DEAE-column was routinely cleaned with 1M NaCl/1% Triton X-100 (v/v) and 0.1M NaOH prior to storage in 0.02% (w/v) azide.

2. Hydroxylapatite Chromatography

Dialysed protein (20-200mg) was routinely loaded on to a 14 x 1.6cm Bio-gel HT (Bio-Rad Laboratories Ltd.) hydroxlyapatite column. The column was pre-equilibrated in buffer D, and the protein material checked for equivalent pH (6.8) and conductivity (13 mS/cm) prior to loading. A flow rate of 15 ml/sq.cm/h was maintained throughout loading, washing and elution. After loading, the column was washed until no further eluting protein was detected at 280nm with buffer D. Elution of the esterase was with a gradient of 30ml each of buffer D to buffer E (identical to buffer D except that 300mM potassium phosphate was used) followed by a wash of 30ml of buffer E. Fractions (3ml) were collected and assayed for cocaine esterase activity. A typical elution profile is shown in Fig. 2. Active fractions were pooled and concentrated against PEG 4000 (20% w/v) to a final volume of

approximately 4ml. (The column was routinely cleaned with 1M NaCl/0.5% (w/v) cholate and stored in 0.02% (w/v) azide).

3. AcA 44 Gel Filtration Chromatography

Cocaine esterase from the previous step was concentrated to approximately 4ml and loaded on to a 60 x 1.6cm column of AcA 44 Ultrogel (Life Science Laboratories Ltd.) which had been pre-equilibrated in buffer B. In the presence of cholate, the cocaine esterase would be expected to elute as a protein with a molecular weight of 110,000 Daltons. Elution with buffer B was performed at a flow rate of 4ml/sq.cm/h and the elute collected as 2ml fractions. Assays for cocaine esterase activity were performed. A typical elution profile is shown in Fig. 3. Active fractions were pooled for cholate removal.

4. Removal of cholate

The pooled enzyme was diluted 1:10 with buffer A (to bring the cholate concentration below the critical micelle concentration) and dialysed against 2 x 10 volumes of the same buffer to remove salt and cholate. Cholate removal was monitored by simply adding 20µl of sample to 1ml of Coomassie dye binding protein assay reagent, which contains phosphoric acid as a major component. Under the acidic conditions, cholate-containing samples formed a vivid blue precipitate due to the precipitation of cholic acid. Post-AcA 44 material treated as described above did not produce this precipitate, even after subsequent concentration, indicating effective removal of cholate.

5. Sephacryl S-300 Gel Filtration Chromatography

Concentrated cholate-free esterase was loaded on to a 75 x 1.6cm column of Sephacryl S-300 (Pharmacia LKB Biotechnology Ltd.) pre-equilibrated in buffer A. Subsequent elution with buffer A was at a flow rate of 4 ml/sq.cm/h. In this cholate-free state the esterase was expected to elute as a protein with molecular weight 410,000 Daltons. Collection and assay of 2ml fractions typically gave a profile as shown in Fig. 4.

Results

In a solubilised extract of P. maltophilia MB11L grown on

cocaine as the carbon source, cocaine esterase was present at a specific activity of 0.16 unit (mg protein)$^{-1}$. It was purified 22-fold as shown in Table 1 below.

EXAMPLE 3

Characterisation of cocaine esterase

pH optimum

Purified cocaine esterase material (10µl, 5µg protein) was incubated for 20 min at 30°C with 2mM cocaine in a range of buffers: 50mM Bis Tris Propane (pH 6.0, 7.0, 8.0, 9.0 and 10.0), 50mM MOPS (pH 6.5, 7.0 and 7.5) and 50mM Bicine (pH 7.5, 8.5 and 9.0). After removal of protein by precipitation with 10µl concentrated phosphoric acid and centrifugation at 13,000 rpm in a microfuge, the benzoic acid concentration in each incubation was determined by HPLC analysis and cocaine esterase activity determined. In each case a control assay was performed containing no enzyme, and the assay calibrated using a 1mM benzoate standard. All assays were performed in duplicate.

Cocaine esterase displayed a pH optimum range from 7 to 9. Tertiary amine buffers appeared to have an inhibitory effect on the esterase activity.

Effect of temperature

Samples of cell-free extract were held at various temperatures for at least 30 min prior to centrifugation. Assays for cocaine esterase activity and protein were compared to original levels. The enzyme was stable up to 30°C, above which enzyme activity fell off rapidly.

A comparison of the thermal stabilities of CE and acetylesterase activities in the purified protein preparation (0.015mg protein) was performed in the presence and absence of cholate. The $t_{½}$ of acetyl esterase was 4.5 min in the presence of cholate and approximately 10 min in its absence. CE had a $t_{½}$ of 2.5 min in the presence of cholate and little instability in its absence.

TABLE_1

| Purification Step | Total volume (ml) | Total activity (U) | Overall recovery % | Total protein (mg) | Specific activity (U/mg) | Overall purification (fold) |
|---|---|---|---|---|---|---|
| Solubilised extract | 150 | 163 | 100 | 1017 | 0.16 | 1.0 |
| DEAE-Sephacel Anion exchange Chromatography | 120 | 98 | 60 | 141 | 0.70 | 4.4 |
| Hydroxylapatite HT | 64 | 57 | 35 | 57 | 1.0 | 6.3 |
| AcA 44 Ultrogel Gel filtration | 28 | 46 | 28 | 20 | 2.30 | 14 |
| S-300 Sephacryl Gel filtration | 23 | 14 | 9 | 4 | 3.50 | 22 |

Kinetic characterisation and substrate specificity of cocaine esterase

Incubations of purified cocaine esterase (1.25µg of protein) were performed in 1ml of 50mM bis-tris-propane buffer, pH 8.0, with substrates over the concentration range 0-2mM (0-1mM for cocaine) at 30°C for 10 min. Reactions were stopped using concentrated phosphoric acid (10µl) to precipitate protein, which was removed by centrifugation at 13,000 rpm in a Microfuge. Rates of substrate hydrolysis were calculated from HPLC analyses of the incubations. Non-enzymic rates of hydrolysis were calculated from control samples containing no enzyme, and the enzyme assays corrected accordingly. Standard samples of the relevant free acids were used to calibrate each assay. From the results it was possible to calculate apparent $K_m$ and $V_{max}$ values for cocaine esterase against each substrate. Lineweaver-Burke and Eadie-Hofstee plots were used to obtain these values (see Fersht, 1985, Enzyme Structure and Mechanism, 2nd Edition, Publishers: W. H. Freeman & Co., Oxford for methods). The mean values are listed in Table 2.

Table 2

| SUBSTRATE | Apparent $K_m$ (mM) | Apparent $V_{max}$ (U/mg) |
|---|---|---|
| Cocaine | 0.36 | 29.5 |
| Ethyl benzoate | 1.89 | 65.0 |
| Ethyl 2-hydroxybenzoate | 1.75 | Not determined |
| Ethyl 3-hydroxybenzoate | No activity | No activity |
| Ethyl 4-hydroxybenzoate | No activity | No activity |

Thus, Michaelis constants obtained using the purified enzyme show that cocaine esterase has a greater affinity for cocaine than ethyl benzoates, but hydrolyses cocaine at a slower rate than ethyl benzoates.

Molecular weight determination

The molecular weight of the native/solubilised enzyme was determined by the method of Andrews (Biochem. J. (1964) 91 222-233). For the solubilised enzyme, measurements were performed on a column of Sephacryl S-200 (1.6 x 75cm). Purified cocaine esterase (2mg protein) was solubilised and mixed with marker proteins and the mixture (total volume 2ml) added to the column. The column was eluted with buffer B at 4ml/cm$^2$/h collecting as 1.3ml fractions. The elution volume of cocaine esterase corresponded to a molecular weight of 110,000 Daltons. The molecular weight of the aggregated cocaine esterase was performed in a similar manner on a Sephacryl S-300 column, but no solubilisation was performed and elution was with buffer A. The elution volume of cocaine esterase corresponded to a molecular weight of 410,000 Daltons.

Molecular weight determination was also performed using SDS-PAGE. The purified cocaine esterase sample ran as a distinct major band corresponding to a molecular weight of 129,000 Daltons when compared to standard proteins using the method of Shapiro et al. (Biochem. Biophys. Res. Comms. (1967) 28 815-820). This similar value to that of the solubilised enzyme implies that any effect of detergent/protein interactions in determining the molecular weight of the solubilised enzyme were minimal.

Native PAGE analysis of purified CE showed a major band of Rf 0.2, the gel was sectioned using a scalpel blade, and incubations containing sections of the gel with 2mM cocaine in buffer were analysed for cocaine esterase activity. HPLC analysis of the incubations confirmed that the region of the gel corresponding to the Rf 0.2 major band, hydrolysed cocaine at a rate higher than the other regions of the gel sampled.

Inhibition studies

Samples of purified cocaine esterase (0.01 U) were incubated with a range of possible inhibitors (1mM) at 30°C for 10 min prior to assaying for activity against cocaine in the usual manner. The possible inhibitors tested were phenylmethyl-

sulphonyl fluoride (PMSF), eserine, para-hydroxymercuribenzoate (pHMB), benzoate and ecgonine methyl ester. Cocaine esterase activity was determined in a control sample containing no inhibitor. The results in Table 3 below show that PMSF causes total inactivation of cocaine esterase activity, whilst the other possible active site inhibitors, eserine and pHMB were relatively ineffective as inhibitors.

Table 3

| INHIBITOR | % ACTIVITY RELATIVE TO CONTROL |
|---|---|
| 1 mM PMSF | 0 |
| 1 mM Eserine | 95 |
| 1 mM pHMB | 86 |
| 1 mM Benzoate | 92 |
| 1 mM Ecgonine methyl ester | 82 |

Activities are relative to that determined with 2mM cocaine in the absence of inhibitor (0.9 U/ml = 100% activity).

Activities of commercial esterases against cocaine

A number of commercially available esterase enzymes were screened for their ability to hydrolyse cocaine to benzoic acid and ecgonine methyl ester by incubation with cocaine (2mM) under conditions reported as optimal for their usual activity by the manufacturer. Hydrolysis of cocaine was monitored by HPLC and the results are listed in Table 4. Control incubations containing no enzyme were performed under each set of conditions to allow accurate assaying of enzyme activity. As none of the enzymes tested showed appreciable activity compared to that shown against their usual substrate, the cocaine esterase from MB11L, appears unique in its ability to attack the benzoate ester linkage of cocaine.

Table 4

Screen of esterases for activity against cocaine

Esterase activity with cocaine was monitored by HPLC as described earlier. Reaction mixtures contained 2mM cocaine in 50mM buffer of pH suited for maximum enzyme activity in each case. The activities of the enzymes against cocaine are compared to their usual substrates in the table below.

| ENZYME | SPECIFIC ACTIVITY (U/mg) AGAINST COCAINE | SPECIFIC ACTIVITY (U/mg) AGAINST USUAL SUBSTRATE |
|---|---|---|
| Porcine pancreatic lipase | 0 | 50 |
| Orange peel acetylesterase | 0 | 6.2 |
| Porcine liver esterase | 0.833 | 20 |
| Horse serum butyryl-cholinesterase | 0.098 | 14 |
| α-chymotrypsin (with calcium) | 0.04 | 40 |
| Electric eel acetyl-cholinesterase | 0 | 320 |
| Cocaine esterase[1] | 3.5 | - |
| Atropinesterase[2] | 0 | 1.5[3] |

[1] Purified enzyme from MB11L.

[2] A sample of crude extract from Psuedomonas putida PMBL-1 grown on atropine as sole carbon source with 0.05U of atropine hydrolysing activity (measured by HPLC) was incubated with 2mM cocaine for 20 min at 30°C. No breakdown of cocaine was seen by HPLC analysis.

[3] This value is from crude extract studies by Stevens ((1969) PhD Thesis, University of Leiden, Netherlands). Purified atropinesterase has a specific activity of 500-600U/mg (Hessing, (1983), PhD Thesis, University of Leiden, Netherlands).

Effect of growth substrate on cocaine esterase activity

MB11L cells were grown in 750ml batch culture on a range of carbon sources (10mM) in medium B. After 3 sub-inoculations cells were harvested and crude extracts prepared. Assays for cocaine esterase and protein were performed and the results expressed relative to levels seen in extracts from the cells grown on cocaine (0.02µmol/min/mg protein = 100%), are shown in Table 5.

Table 5

| ENZYME | RELATIVE ENZYME ACTIVITY GROWTH SUBSTRATE Benzoate | Citrate | Cocaine | Glucose |
|---|---|---|---|---|
| Cocaine esterase | 20% | 0% | 100% | 20% |

These results clearly demonstrate the low levels of cocaine esterase from cells grown in the absence of cocaine (cells grown on benzoate or glucose give extracts possessing only 20% of the specific activity of cells grown on cocaine, whilst citrate grown cells possess no activity), indicating that cocaine esterase is inducible. Inclusion of 40mg/l D,L-methionine in the growth medium increased the specific activity of the cocaine esterase produced by 20%.

The following claims define some important aspects of the invention, but do not purport to include every conceivable aspect for which protection might be sought in subsequent continuing and foreign patent applications, and should not be construed as detracting from the generality of the inventive concepts hereinbefore described.

International Application No: PCT/ /

## MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 3, line 25 of the description [1]

**A. IDENTIFICATION OF DEPOSIT [2]**

Further deposits are identified on an additional sheet ☐ [3]

Name of depositary institution [4]

THE NATIONAL COLLECTIONS OF INDUSTRIAL AND MARINE BACTERIA LTD.

Address of depositary institution (including postal code and country) [4]

23 St. Machar Drive, Aberdeen, AB2 1RY

| Date of deposit [4] | Accession Number [4] |
|---|---|
| 9 July 1991 | 40427 |

**B. ADDITIONAL INDICATIONS [5]** (leave blank if not applicable). This information is continued on a separate attached sheet ☐

In respect of all designated states in which such action is possible and to the extent that it is legally permissible under the law of the designated state, it is requested that a sample of the deposited micro-organism(s) be made available only by the issue thereof to an independent expert, in accordance with the relevant patent legislation e.g. EPC Rule 28(4), UK Rule 17(3), Australian Regulation 3.25(3) and generally similar provisions mutatis mutandis for any other designated state.

**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [6]** (if the indications are not for all designated States)

**D. SEPARATE FURNISHING OF INDICATIONS [7]** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [8] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

**E.** ☑ This sheet was received with the international application when filed (to be checked by the receiving Office)

Joanne Burton
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau [10]

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

CLAIMS

1. A cocaine esterase enzyme characterised in that:

(1) it catalyses the debenzoylation of cocaine;

(2) it has a molecular weight in the unaggregated form of about 120,000 Daltons as determined by gel filtration;

(3) it has esterase activity specifically at the benzoate ester linkage of cocaine.

2. A cocaine esterase enzyme according to claim 1 further characterised by one or more of the following features:

(4) it separates at a major band of Rf about 0.2 on PAGE, in its aggregated form;

(5) its unaggregated form is thermally denatured, with a half life of about 2½ minutes at 47°C;

(6) it is inhibited specifically by phenylmethylsulphonyl fluoride.

3. A Pseudomonas maltophilia bacterial strain referred to as "MB11L" and deposited as NCIMB 40427, and mutants and variants thereof capable of producing a cocaine esterase enzyme which degrades cocaine.

4. A process of producing an enzyme according to claim 1 which comprises culturing the Pseudomonas sp NCIMB 40427 or a mutant or variant thereof, according to claim 3, together with a source of carbon and nitrogen, at a temperature of 20 to 40°C, disrupting the cells and recovering the enzyme from the disrupted cells.

5. A method of detecting cocaine in a sample, comprising subjecting the sample to a debenzoylation reaction in the presence of a cocaine esterase according to claim 1 or 2, until ecgonine methyl ester and benzoic acid are produced, and detecting the occurrence of said reaction.

6. A method according to claim 5 wherein benzoate ions liberated in the debenzoylation are detected.

7. A method according to claim 6 wherein the benzoate ions are detected conductimetrically.

8. A conductimetric biosensor comprising a pair of electrodes in contact with an electrolyte containing a cocaine esterase

according to claim 1 or 2.

9. A sensor comprising a cocaine esterase according to claim 1 or 2, in working relationship with a potentiometric, amperometric, conductimetric or piezoelectric transducer.

10. The use of a cocaine esterase enzyme as defined in Claim 1 or 2, in therapy, biotransformation or cocaine waste treatment.

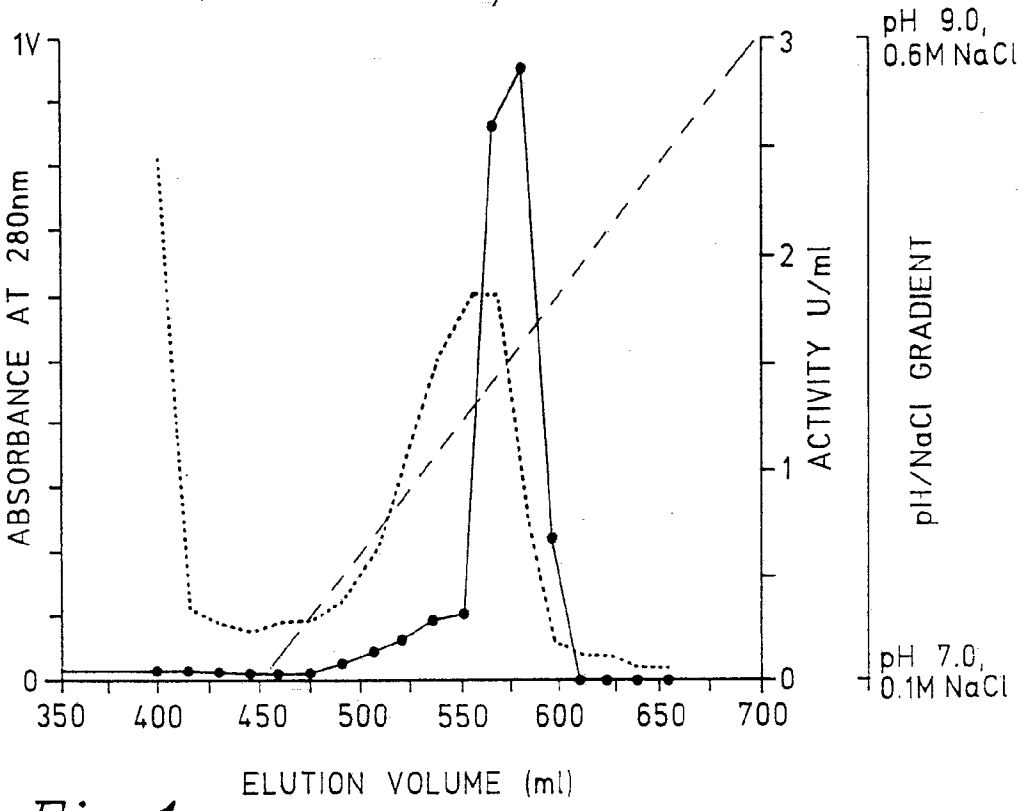
1V
ABSORBANCE AT 280nm
0
3
2
1
0
ACTIVITY U/ml
pH 9.0,
0.6M NaCl
pH/NaCl GRADIENT
pH 7.0,
0.1M NaCl
350
400
450
500
550
600
650
700
ELUTION VOLUME (ml)

*Fig. 1*

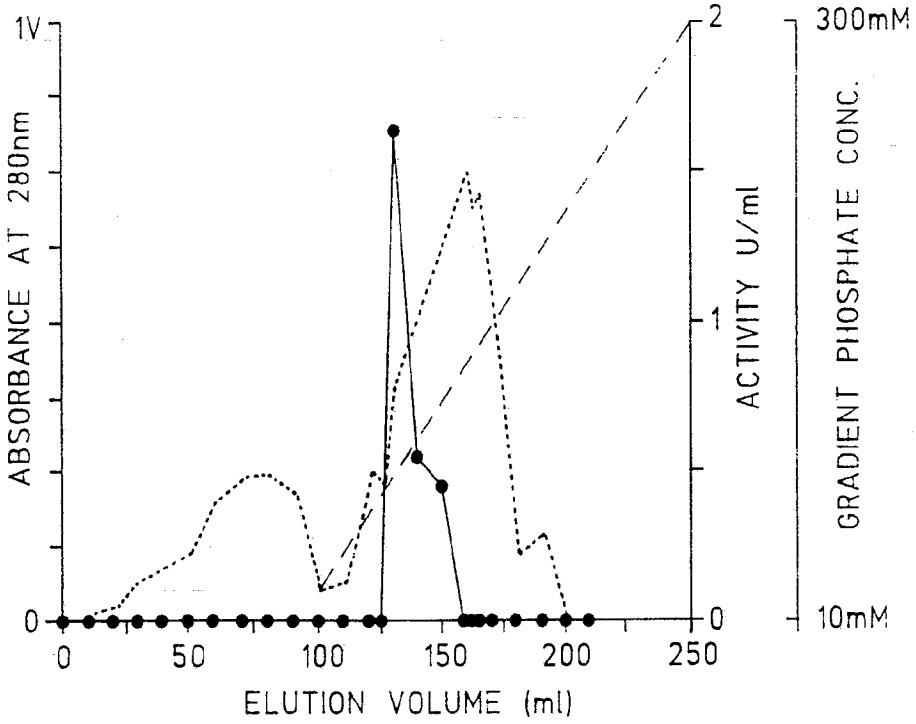
1V
ABSORBANCE AT 280nm
0
2
1
0
ACTIVITY U/ml
300mM
GRADIENT PHOSPHATE CONC.
10mM
0
50
100
150
200
250
ELUTION VOLUME (ml)

*Fig. 2*

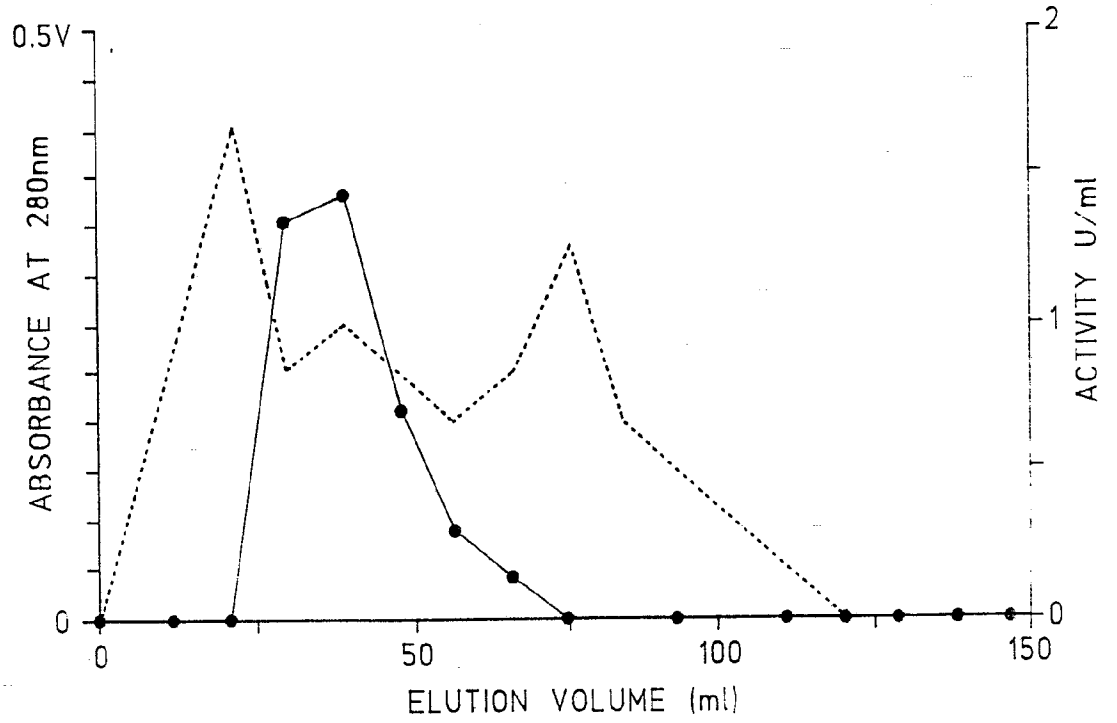
0.5V
ABSORBANCE AT 280nm
0
0
50
100
150
ELUTION VOLUME (ml)
2
1
0
ACTIVITY U/ml

*Fig.3*

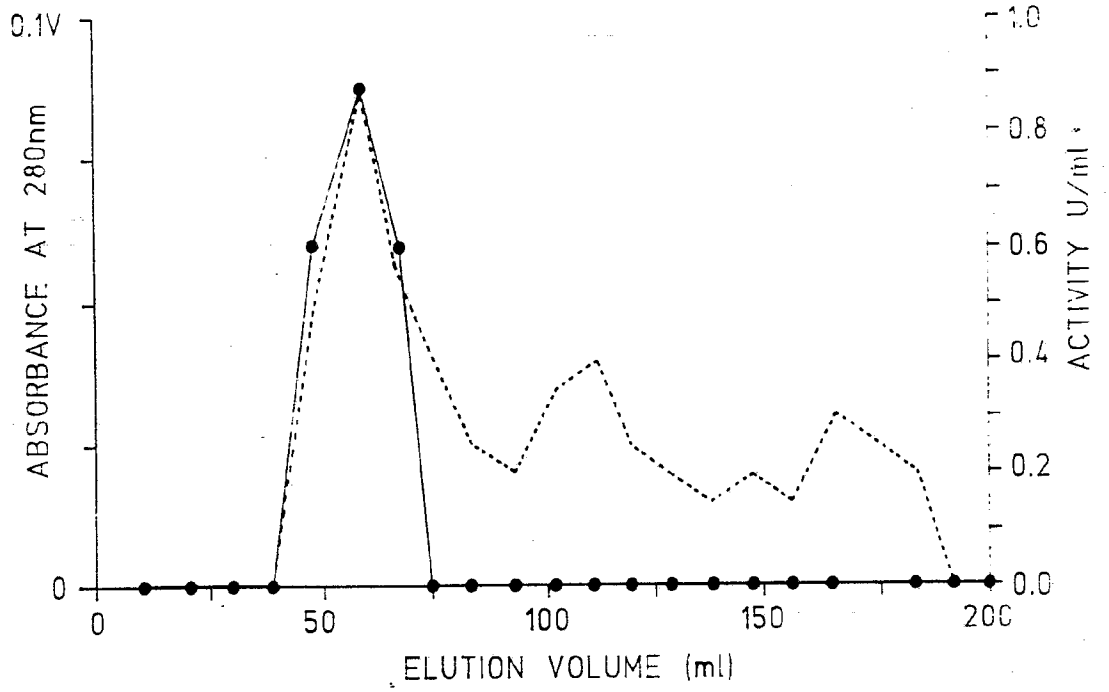
0.1V
ABSORBANCE AT 280nm
0
0
50
100
150
200
ELUTION VOLUME (ml)
1.0
0.8
0.6
0.4
0.2
0.0
ACTIVITY U/ml

*Fig.4*

# INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/01324

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N9/18; C12N1/20; C12Q1/44; A61K37/54
C12P17/18; C12M1/40; //(C12N1/20;C12R1:38)

## II. FIELDS SEARCHED

Minimum Documentation Searched[7]

| Classification System | Classification Symbols |
|---|---|
| Int.Cl. 5 | C12N |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

| Category ° | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No.[13] |
|---|---|---|
| X | FASEB JOURNAL. vol. 5, no. 5, 15 March 1991, BETHESDA, MD US page A1205 R. DEAN ET AL 'Cocaine metabolism to benzoylecgonine, ethylcocaine and ecgonine methyl ester by human liver extracts' see abstract --- | 1,2,10 |
| X | FASEB JOURNAL. vol. 5, no. 12, September 1991, BETHESDA, MD US pages 2735 - 2739 R. DEAN ET AL 'Human liver cocaine esterases: ethanol-mediated formation of ethylcocaine' see the whole document especially page 2737 --- -/-- | 1,2,10 |

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 28 OCTOBER 1992 | 24. 11. 92 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | VAN DER SCHAAL C.A. |

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

| Category ° | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No. |
|---|---|---|
| Y | WO,A,193 634 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) 15 November 1990 see page 8, line 15 - page 10; examples 5,6 | 5-9 |
| P,X | CHEMICAL ABSTRACTS, vol. 116, no. 22, 1 June 1992, Columbus, Ohio, US; abstract no. 210040, A.J. BRITT ET AL 'Identification of a cocaine esterase in a strain of Pseudomonas maltophilia' page 308 ; see abstract | 1-4,10 |
| Y | & JOURNAL OF BACTERIOLOGY vol. 174, no. 7, 1992, pages 2087 - 2094 | 5-9 |

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9201324 SA 62475

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 28/10/92
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO-A-193634 | | None | |